Award Number: W81XWH-10-1-0689

TITLE: Role of the Rb and p53 Tumor Suppressor Pathways in Mammary Tumorigenesis

PRINCIPAL INVESTIGATOR: Robert A. Jones, Ph.D.

CONTRACTING ORGANIZATION:
University Health Network
Toronto, ON, Canada
M5G 2M1

REPORT DATE: September 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
1 September 2012	Annual Summary	Sept 1 2011-Aug 31 2012

4. TITLE AND SUBTITLE
Role of the Rb and p53 Tumor Suppressor Pathways in Mammary Tumorigenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0689

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert A. Jones, Ph.D.

robertj@uhnres.utoronto.ca

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Toronto General Research Institute, University Health Network
Toronto, Ontario M5G 2M1

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Understanding the genetic pathways that contribute to the development of specific breast cancer subtypes will lead to the discovery of more effective therapies. Two of the most common genetic alterations in human cancers involve inactivation of the Rb and p53 tumor suppressor pathways. To directly investigate the role of these pathways in breast cancer, we generated mice with conditional deletion of Rb and p53 in the mammary epithelium. Combined loss of Rb and p53 led to the development of tumors with features of epithelial-mesenchymal-transition (EMT) that are enriched for tumor-initiating activity. Microarray analysis revealed these tumors are similar to EMT-type tumors from other mouse mammary tumor models and the claudin-low subtype of human breast cancer. We have now begun using high-throughput chemical screens and and whole-genome lentiviral shRNA libraries to screen Rb/p53 deficient primary mouse mammary tumor cell lines for novel therapeutic targets.

15. SUBJECT TERMS
Mouse mammary, tumor suppressor, EMT, tumor-initiating cell, cell of origin, shRNA screen, chemical screen

16. SECURITY CLASSIFICATION OF: Unclassified			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	21	USAMRMC
					19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION

Alterations in the Rb and p53 tumor suppressor pathways are commonly observed in human cancers including breast cancer. Our lab has previously reported that targeted deletion of Rb in mouse mammary progenitors leads to heterogeneous tumors including luminal-B and basal-like/EMT (epithelial-to-mesenchymal transition) subtype tumors (1). Interestingly, the basal-like/EMT but not luminal-B subtype tumors often contained p53 mutations. Accordingly, targeted deletion of Rb and p53 in the mammary epithelium led to the development of EMT-type tumors which expressed high levels of the mesenchymal marker N-cadherin and low levels of the epithelial marker cytokeratin 8. The experiments in this proposal seek to further characterize mammary tumors induced following combined loss of Rb and p53. Specifically, we will use gene expression profiling to identify the molecular subtype of Rb/p53 deficient mammary tumors and use FACS sorted cells transplanted at limiting dilutions to investigate whether these tumors contain a tumor-initiating cell (TIC) population. In addition, we will develop a strategy to delete Rb and p53 in specific cell populations within the mammary gland to determine whether the cellular origin of the disease impacts the tumor phenotype. Finally, we will employ a powerful shRNA screening technology to identify new therapeutic targets for breast cancer.

BODY

Task 1. *Characterize mammary tumors in Rb/p53 conditional knockout mice and identify tumor-initiating cells (TICs)*

a) Generate additional MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ and MMTV-Cre:$p53^{fl/fl}$ conditional knockout mice and monitor for tumor development. ***In progress***

Previous Results: Mice lacking both Rb and p53 in the mammary gland were viable and fertile; however the majority of animals developed lethal lymphomas or died of unknown causes (possibly other hematological disease) by 2-6 months of age. This is due to expression of MMTV-Cre in cellular compartments other than the mammary epithelium. To circumvent this, $1x10^4$-$1x10^5$ primary mammary epithelial cells (MECs) isolated from MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ mice were transplanted into the mammary fat pads of NOD/SCID or RAG1-/- mice and monitored for tumor development. A total of 5 independent tumors (ie. 5 tumors originating from 5 separate donors) have been collected and we are monitoring additional animals for tumor development. In addition, I am monitoring a cohort of MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ animals (both virgin and multiparous) that have not yet developed lymphomas or other malignancies. This will provide us with a large number of independent tumor samples for future microarray and aCGH analysis.

All of the tumors collected to date are comprised primarily of elongated spindle-shaped cells with some tumors also containing small clusters of epithelial cells. These histological findings are suggestive of an epithelial-to-mesenchymal transition (EMT). Indeed, the first two tumors collected and analyzed by immunohistochemistry expressed high levels of the mesenchymal markers N-cadherin and desmin while expressing low levels of the epithelial marker cytokeratin 8. This initial analysis of Rb/p53 deficient mammary tumors (also termed ΔRb/p53) was published in the *Journal of Clinical Investigation* (1). The cohorts of mice still being monitored for tumor development as described above will provide a more complete picture of tumor onset and pathology of Rb/p53 deficient tumors.

New Results: As described above, we found that transplantation of primary MECs isolated from MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ into the cleared fat pads of immunodeficient mice led to the development of mammary tumors with characteristics of EMT. In an attempt to generate additional Rb/p53 deficient mammary tumors, I also transplanted $Rb^{fl/fl}$:$p53^{fl/fl}$ cells that had been infected with a Cre expressing Adenovirus (Ad-Cre). This experiment also resulted in the development of EMT-type tumors. Finally, I also collected mammary tumors from 8 MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ mice (7 virgin, 1 multiparous) without the use of transplantation. Similar to the tumors obtained from transplantation experiments the majority (7/8) of these lesions can be categorized as EMT-type tumors. This data is illustrated in Fig. 1. Importantly, the formation of tumors with an EMT phenotype in the absence of transplantation provides evidence that the transplantation procedure itself (enzymatic digestion of mammary tissue to single cells) does not promote the formation of EMT-type tumors. However, the median latency of tumor development was slightly longer in the absence of transplantation (median time to tumor development of 282 days compared to 245 and 231 days).

b) Verify deletion of Rb and p53 in mammary tumors. ***In progress***

Previous and New Results: Now that we have accumulated a significant number of tumors, PCR analysis of each sample will be carried out in the final year.

c) Determine molecular subtype(s) of Rb/p53 and p53 deficient mammary tumors by histological/immunohistological staining, RNA expression microarray, DNA CGH analysis. ***In progress***

Results: During this year past year of the project we collected a sufficient number of tumors to be analyzed at the molecular and genetic level. For microarray analysis, the samples included 10 ΔRb/p53 mammary tumors, 3 cell lines derived from ΔRb/p53 tumors and 5 age-matched mammary glands isolated from $Rb^{f/f}$;$p53^{fl/fl}$ mice. In addition, 1 EMT-type p53 deficient tumor was included for analysis (Dr. Jeff Liu, a postdoctoral fellow in the lab, has collected additional MMTV:$p53^{fl/fl}$ tumors), as well as 1 MMTV-Neu tumor (a luminal-type tumor) as a control. The samples were processed using the Affymetrix platform (Mouse Gene 1.0 ST Chip) with analysis performed in collaboration with Dr. Liu using Partek Genomics Suite.

As expected, cluster analysis of the samples revealed that each tissue/tumor type clustered into discreet groups (Fig. 2A). To investigate gene expression changes associated with tumorigenesis, we compared the expression profiles of ΔRb/p53 tumors, ΔRb/p53 cell lines and WT mammary glands (Fig. 2B). This analysis revealed that 4009 genes were significantly altered at least 2-fold between ΔRb/p53 tumors and normal mammary tissue at least 2-fold (2078 up-regulated, 1931 down-regulated). Additionally, we also identified 7009 genes whose expression was altered between ΔRb/p53 cell lines and WT mammary glands (2078 up-regulated and 36871 down-regulated). Due to the ease with which cell lines can be manipulated in vitro to study tumorigenesis (compared to in vivo using genetically engineered mouse models), we also generated a list of differentially expressed genes that were shared by both ΔRb/p53 tumors and ΔRb/p53 cell lines in comparison to WT mammary glands. A total of 2575 genes were found to be differentially expressed (1175 up-regulated, 1400 down-regulated) and the top 5 from each category are summarized in Fig. 2C. In the future, selected genes can be manipulated in the cell lines in vitro using knockdown (upregulated genes) or overexpressed (downregulated genes) to study their function in tumor development and progression.

Another goal of this objective is to determine the molecular subtype of Rb/p53 deficient mammary tumors. To address this, we carried out unsupervised hierarchical clustering of ΔRb/p53 tumors with the panel of mouse mammary tumor samples from Herschkowitz et al. (2007). This analysis revealed that Rb/p53 deficient tumors clustered most closely with a subset of tumors from the DMBA, MMTV-Cre;$Brca1^{fl/fl}$;p53+/-, p53 null, p53+/-, and Wap-Tag models (Fig. 3). Similar to ΔRb/p53 tumors, these samples were categorized as spindle cell tumors based on histopathology and express high levels of EMT associated genes. Given that EMT-type tumors in mice correspond to the claudin-low subtype of human breast cancer, we also analyzed ΔRb/p53 tumors using the Prat et al. (2010) claudin-low predictor. Indeed, the Rb/p53 deficient tumors were found to cluster with human claudin-low breast cancers (Fig. 4).

To complement the analysis of the global gene expression changes within Rb/p53 deficient tumors, we are now performing Gene Set Enrichment Analysis (GSEA) in collaboration with Dr. Veronique Vousin of the Ontario Institute for Cancer Research). GSEA is a powerful computation method used to identify sets of genes (biological pathways) that are altered between two sample groups. In addition to comparing primary tumors to normal mammary tissue, we will also compare ΔRb/p53 tumors to Δp53 (single knockout) tumors. While loss of p53 alone leads to the development of heterogeneous tumors, we have collected a number of Δp53 (single knockout) classified as claudin-low. Therefore, this comparison will determine whether claudin-low tumors can be subdivided into additional subgroups depending on the genetic lesions present within the tumor.

Finally, we also proposed to examine the genomic changes in Rb/p53 deficient tumors using high density aCGH. A total of 10 ΔRb/p53 tumors have been processed at the Toronto Centre for Applied Genomics (TCAG) on the Agilent platform (1M chip). The data for this experiment is currently being analyzed in collaboration with Dr. Lakshmi Muthuswamy of the Ontario Institute for Cancer Research.

d) Isolate and characterize tumor initiating cells in MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ mice by FACS analysis and limiting dilution transplantation into the mammary fat pad of NOD/SCID mice. ***Completed***

Previous Results: There is growing but incomplete evidence that human cancers may be organized as a hierarchy in which only a subpopulation of cells within a tumor have tumor-initiating capabilities (the cancer stem cell hypothesis) (4). In the mouse, mammary gland stem cells have been identified using FACS sorted cells in combination with limiting dilution transplantation assays. These cells are identified as $CD49f^{+}CD24^{+}$ or

$CD29^+CD24^+$ and can give rise to a complete and functional mammary gland (5, 6). Tumor-initiating cells have also been identified using a variety of cell surface markers in mouse models of breast cancer including MMTV-Neu, MMTV-Wnt and p53 null tumors (7, 8, 9).

TIC analysis was carried out on freshly isolated cells from a total of 4 Rb/p53 deficient mammary tumors. The majority of tumors contained both a $CD49^+$ single positive and $CD49^-CD24^-$ double negative population while the remaining tumor displayed a predominant $CD49^+CD24^+$ double positive population. Transplantation of FACS sorted cells at limiting dilutions into the mammary fat pads of NOD/SCID mice revealed that Rb/p53 deficient mammary tumors are highly enriched for tumor-initiating capacity. This is in agreement with a recent study from the Rosen lab in which they showed p53 null EMT-type tumors contained a greater number of TICs compared to adenocarcinomas from the same model (10). However, in our model of EMT-type tumors, TICs are enriched in both the $CD49^+$ single positive and $CD49^-CD24^-$ double negative population while TICs in the p53 null model are enriched in the $CD29^+$ single positive and $CD29^+CD24^+$ double positive population. Thus, even though both Rb/p53 and p53 deficient EMT-type tumors share similar histological characteristics and are enriched for TICs, the FACS profiles of these tumors are distinct suggesting that there is molecular heterogeneity within this tumor subtype.

New Results: Over the past year I carried out TIC analysis on an additional Rb/p53 deficient mammary tumor. Similar to the other 4 experiments, this tumor showed enrichment for TICs in the CD49+ and CD49-CD24- populations. A table summarizing TIC activity in ΔRb/p53 tumors is shown in Fig. 5.

e) Generate a gene expression signature of tumor- initiating cells and determine prognostic significance.
Results: As per the Statement of Work, these experiments will be carried out in the 3rd year of funding.

Task 2. *Characterize mammary tumor development following Rb/p53 deletion in different mammary cell populations*
a) Generate $Rb^{f/f}$:$p53^{f/f}$ conditional knockout mice lacking the MMTV-Cre transgene. ***Completed***
Results: A cohort of mice containing the $Rb^{fl/fl}$:$p53^{fl/fl}$ alleles (*without* MMTV-Cre) have been generated. In addition, some of these mice have been crossed with the Rosa-26 reporter strain to permanently mark recombined cells in vitro and in vivo.

b) Isolate stem, luminal and myoepithelial cells/progenitors by FACS from $Rb^{fl/fl}$:$p53^{fl/fl}$ mice, infect with Lenti-Cre-EGFP and transplant into the mammary fat pad of NOD/SCID mice. ***In progress***
Previous Results: The goal of this objective is to investigate whether Rb/p53 deficient mammary tumors develop from a particular cell population within the mammary gland (stem, luminal or myoepithelial cell) and/or whether the cell of origin influences the phenotype of the tumor. I initially proposed to use a Lenti-Cre-EGFP vector to delete the $Rb^{f/f}$ and $p53^{f/f}$ alleles in vitro. However, due to the expertise and time required to generate high titer lentivirus, especially with larger constructs such as Lenti-Cre-EGFP, I have opted to use a commercially available Adenovirus for these experiments (Ad-Cre-IRES-GFP or Ad-Cre, Vector BioLabs). Also, given that constitutive Cre expression has been shown to be toxic in some cell types, the use of an adenovirus may be more advantageous than a lentivirus based system since adenoviruses do not integrate into the host genome and the Cre transgene will only be expressed transiently.

To determine the percentage of primary MECs that can be infected with Ad-Cre, cells were incubated with virus under low attachment conditions at a range of MOIs and analyzed for GFP expression by FACS after 48 hrs. In the initial experiment, approximately 30-40% of MECs expressed GFP and recombination of the conditional alleles was readily detectable by PCR. More recently, I have further optimized the protocol to routinely achieve between 60% and 80% infection of primary MECs.

Initially, I intended to use FACS to separate the 3 cell populations that can be delineated in the mammary gland using CD49 and CD24 cell surface markers (luminal, stem and myoepithelial). However, due to the small number of cells located within the stem cell fraction (located at the tip of the basal cell population) and the lack of purity of the this population (contamination with myoepithelial cells, 5, 6), I have decided to sort and infect the two major populations ($CD24^+$ luminal and $CD49^+CD24^+$basal cells). In a preliminary experiment carried out to optimize the number of mammary glands required to obtain a sufficient number of cells, as well as test the sorting and culture conditions, I was able to observe clear differences between the luminal and basal fractions when cultured under low attachment conditions. The luminal population tends to aggregate together but remain as single cells while the basal population tends to form tight spheres that have a

'glassy' appearance. As the conditions are now optimized, these experiments will be carried out during the 2nd year of funding.

New Results: As described above, in preliminary experiments, basal and luminal cells were isolated by FACS and cultured under low attachment conditions. This culture method was chosen due to the ease with which to recover the cells after overnight infection with Ad-Cre (compared with unknown effects of monolayer culture on differentiation status and use of trypsin to isolated single cells). However, while a large fraction of basal cells remained viable the next day, trypan blue staining of the luminal fraction indicated that most of these cells had died. Therefore, to enhance cell viability, I have begun culturing the sorted cells under 3D conditions. It has been shown previously that FACS sorted mammary basal cells form solid organoids while luminal cells form hollow acinar structures when cultured in Matrigel. Traditional 3D culture methods involve culturing the cells either on a thin layer of Matrigel ('on-top' assay) or between two layers of Matrigel ('embedded' assay). However, one of the major limitations of these methods is the difficulty with which to retrieve the cells for further experimental manipulation. Therefore, I have utilized a modified Matrigel culture method that was recently reported by the Weinberg lab (11). This modified assay uses a reduced Matrigel concentration of 5% (versus 100%) to facilitate recovery of the cells while still enabling the basal and luminal cell populations to form distinct organoids. In agreement with Guo *et al.* (2012), I have found that sorted luminal and basal cells form acinar and solid organoids respectively under these culture conditions and are capable of being infected with Ad-Cre-IRES-GFP (Fig. 6B). Therefore, to investigate the cellular origin of Rb/p53 deficient tumors I have developed the following protocol illustrated in Fig. 6A. Briefly, primary MECs were isolated from $Rb^{f/f}$:$p53^{f/f}$ mice and sorted by FACS into luminal and basal populations. The cells are then cultured in the modified Matrigel assay for 5-8 days to allow for organoid formation. The organoids are then collected and dissociated into single cells by trypsinization, counted and infected with Ad-Cre-IRES-GFP at an MOI of 50 for 1-2hrs in suspension. The cells are then washed, counted, resuspended in a 1:1 ratio of HBSS and Matrigel and injected into the cleared fat pads of 3 week old immunodeficient mice. Previous reports have demonstrated that transplantation of luminal cells in the absence of supporting basal cells rarely leads to ductal outgrowths (due to a lack of stem cell activity) (5,6 12). Thus, the infected cells are also mixed with an equal number of wild type MECs (without floxed alleles and isolated from FvB or C57/Bl6 mice) containing both luminal and basal cells to promote engraftment of the luminal population.

c) Characterize mammary tumors by H&E, immunohistochemistry and microarray analysis. ***In Progress***

Results: Despite the challenging nature of this experiment I have already obtained some interesting results. One of the hypotheses regarding the cellular origin of breast cancer suggests that while luminal and basal-like tumors originate from luminal progenitor cells, claudin-low tumors, which express stem cell associated genes, may arise from a basal/stem cell. Therefore, it is interesting that I have observed tumors from both Rb/p53 deficient luminal and basal cells and these tumors have an identical EMT histology (Fig.6B). Control experiments utilizing wild type cells are in progress to ensure Ad-Cre infection itself does not promote tumorigenesis. Nevertheless, this data suggests that at least in the context of combined Rb/p53 deficiency, the genetic the cell of origin does not dictate tumor phenotype.

Given this exciting and somewhat surprising result, I am planning on conducting some additional experiments to investigate the origin of Rb/p53 deficient tumors. For instance, although these results suggest that both luminal and basal cells can give rise to claudin-low tumors in the context of Rb/p53 deficiency, it is unclear whether both cell types have a similar potential for transformation. To address this I will transplant limiting dilutions of each Rb/p53 deficient cell type and quantify the number of tumors that arise. In addition, we and others have previously reported that loss of Rb or p53 alone leads to the development of heterogeneous mammary tumors. Thus, it would be interesting to investigate whether the heterogeneous nature of these tumors (as opposed to homogenous claudin-low tumors associated with combined RB/p53 loss) is dependent/independent of the cell of origin.

Task 3. *Identify therapeutic targets in Rb/p53 deficient mammary tumor cells*

a) Isolate at least 2 additional primary Rb/p53 deficient mammary tumor cell lines ***Completed***

Previous Results: The cell lines isolated from Rb/p53 deficient primary tumors are termed TMCRP3, TMCRP 1, TMCRP 13 and RB544. All of these lines display a spindle cell morphology similar to the primary tumors from which they were derive. Both TMCRP1 and TMCRP 13 lines carry the recombined Rb and p53 alleles and

lack Rb and p53 protein expression while TMCRP3 and RB544 cells were derived from a tumor which lacked Rb but contained a p53 mutation.

b) Perform lentiviral shRNA in vitro whole genome screens on 3 independent tumor cell lines using HC11 normal mouse mammary epithelial cell line as a control. ***In progress***

Results: Genome wide shRNA screens on 2 of the 3 Rb/p53 deficient tumor cell lines and optimized the conditions for the other tumor line as well as HC11 cells. The optimization procedure consisted of calculating cell line doubling time, seeding density, polybrene (used to enhance viral transduction) and puromycin (used to select transduced cells) sensitivity and MOI calculation. To calculate MOI, cells were mixed with a dilution series of pooled virus (1.0ml, 0.75ml, 0.5ml, 0.25 ml) and seeded in duplicate 15cm plates. The following day, culture media of one dilution series replaced with puromycin containing medium while the other dilution series was given fresh media without puromycin. After 48hrs, cells were collected and the MOI (+/- puromycin). An MOI of 0.3-0.4 was used to ensure only 1 shRNA is expressed per cell. For the actual screen, a total of at least $53x10^6$ cells were infected with the pooled virus to give a representation of ~200 cells/shRNA. The cells were split into 3 replicates of $\sim 20x10^6$ cells following the first passage and each cell line was passaged out for approximately 3 weeks. At each passage, $\sim 30x10^6$ cells were frozen down for DNA extraction and subsequent microarray hybridization.

New Results: For technical reasons, no major progress has been made on this objective during the past year. First, although conditions for the 3rd tumor cell line and HC11 control cell line appeared to be optimized, I had difficulties obtaining the correct MOI during the actual screening procedure. Specifically, I have not been able to achieve the appropriate MOI after puromycin selection. After discussing the results with members of the COLT (CCBR-OICR Lentiviral Technology) facility, it was determined that the titer of the pooled virus was significantly reduced even after a single freeze-thaw. Since I had also used freeze-thawed virus for the other 2 cell lines it was determined that the screens should be repeated with fresh virus. I recently obtained new aliquots of pooled virus and will carry out the screens during the 3rd year.

Due to the technical issues associated with the pooled shRNA screen, we decided to carry out 2 high-throughput chemical screens as an alternative strategy to identify potential therapeutic targets for Rb/p53 deficient breast cancer. The first screen involved a library (obtained from Ontario Institute for Cancer Research) of 264 small molecule kinase inhibitors. Two Rb/p53 deficient tumor cell lines and the normal mammary epithelial cell line, HC11, were screened at a final inhibitor concentration of 3uM. The data for the 2 tumor cell lines is shown in Figure 7. The results of this screen revealed a number of interesting 'hits' that were common to both tumor cell lines including SYK kinase. Validation of 'hits' from the screen is currently underway.

The second chemical screen was carried out in collaboration with Dr. Aaron Schimmer (Ontario Cancer Institute) who provided us with a library of 312 FDA-approved drugs. This library, also called a repurposing library, contains a number of off-patent drugs including chemotherapeutics, anti-fungals and anti-microbials. Since these 'old drugs' are already approved for use in various diseases, they can be rapidly moved into clinical trials. The screen was performed at a high (10uM) and low (1uM) concentration on 3 Rb/p53 deficient mammary tumor cell lines and the data is summarized in Figure 8. Some of the 'hits' included chemotherapeutic agents (eg. Fludarabine) that are currently used for the treatment of other cancer types such as leukemia. In addition, a number of anti-microbials (eg. Salinomycin) were found to have anti-cancer activity. Validation of this screen is also currently in progress.

c) Perform lentiviral shRNA in vivo kinase screen using 3 independent tumor cell lines

Previous Results: As described above, the conditions to carry out large scale negative selection screens have been optimized. Once the in vitro screen is completed, the in vivo screen will be carried out.

New Results: No new progress has been made on this part of the objective as a result of the technical issues associated with the in vitro screen.

d) Validate at least 10 'hits' from in vitro screen by MTT survival assay and in vivo screen by transplantation, validate shRNA knockdown by Western blotting

Results: No progress has been made towards this part of the objective due to the technical hurdles encountered with the primary shRNA screen. However, as described above, we have carried out 2 high-throughput chemical screens. Unlike the off-patent screen in which the target of the drug is not always known, the small molecule screen consisted of known targets (kinases). Thus, while the shRNA screens are being

repeated I can validate targets identified in the kinase screen as proposed (MTT assay, western blotting etc.) using a combination of small molecules and shRNAs.

e) Validate at least 5 'hits'from in vitro screen in vivo by transplanting lenti-shRNA infected Rb/p53 deficient tumor cells as well as tet-inducible shRNA into the mammary fat pad of NOD/SCID mice and monitor tumor kinetics

N/A during this year of funding

TRAINING

During the past year I attended the Second Annual Rb Meeting in Toronto and the New Genomic Approaches for Gene Discovery and Personal Treatment in Cancer Workshop at the University of Toronto. I have also participated in weekly laboratory meetings (joint meetings with the laboratory of Dr. Sean Egan, Hospital for Sick Children), local seminars (held weekly at Princess Margaret Hospital focused on breast cancer research) and trained and supervised an undergraduate project student and a volunteer who went on to obtain employment as a laboratory technician at UHN.

KEY RESEARCH ACCOMPLISHMENTS

- Found that MMTV:Cre:$Rb^{f/f}$;$p53^{f/f}$ mice develop EMT-type tumors even in the absence of transplantation
- Carried out microarray analysis on Rb/p53 deficient mammary tumors and found that MMTV:Cre:$Rb^{f/f}$;$p53^{f/f}$ mice model the claudin-low subtype of human breast cancer
- Optimized conditions for the growth of FACS sorted luminal and basal cells as organoids using 3D culture conditions
- Discovered that deletion of Rb/p53 in luminal or basal cells leads to the development of EMT-type tumors
- Carried out 2 high-throughput chemical screens on Rb/p53 deficient mammary tumor cell lines (kinase screen and off-patent drug screen)

REPORTABLE OUTCOMES

Jones, Robert., Jiang, Zhe., Deng, Tao., Schimmer, AD., Moffat, J and Zacksenhaus, E. Role of the RB and p53 tumor suppressor pathways in mammary tumorigenesis. 2011 Second Internatinal Rb Meeting, Toronto, Canada

Jones, Robert., Jiang, Zhe., Deng, Tao., Schimmer, AD., Moffat, J and Zacksenhaus, E. Role of the RB and p53 tumor suppressor pathways in mammary tumorigenesis. CDMRP 2011 Era of Hope Conference

Jiang Z, **Jones R**, Liu JC, Deng T, Robinson T, Chung PE, Wang S, Herschkowitz Jl, Egan SE, Perou CM, Zacksenhaus E. RB1 and p53 at the crossroads of EMT and triple-negative breast cancer. *Cell Cycle* 2011 May 15; 10(10): 1563-1570.

Jiang Z, Deng T, **Jones R**, Li H, Herschkowitz JI, Liu JC, Weigman VJ, Tsao MS, Lane TF, Perou CM, Zacksenhaus E. Rb deletion in mammary progenitors induces luminal-B or basal-like/EMT tumor subtypes depending on p53 status. *J Clin Invest* 2010 Sept 1;120(9):3296-3309.

CONCULSIONS

We have found that targeted deletion of two major tumor suppressor pathways in the mammary gland, Rb and p53, leads to the formation of tumors with features of EMT (epithelial to mesenchymal transition). Microarray analysis revealed these tumors are similar to EMT-type tumors found in other mouse models of breast cancer and cluster closely with the Claudin-Low subtype of human breast cancer. Claudin-low tumors are a subtype of

'triple-negative tumors' which have been shown to be enriched for stem cell-like properties. Indeed, we found that Rb/p53 deficient mammary tumors are enriched for tumor-initiating capacity compared to other mouse models of breast cancer. Thus, our mouse model of Rb/p53 deficient breast cancer will be an invaluable tool to identify and test therapies targeting tumor-initiating cells and claudin-low tumors.

We have also begun to examine the cell of origin of Rb/p53 mammary tumors. Experimental conditions have been optimized that allow us to delete conditional alleles within the different subpopulations of epithelial cells that make up the mammary gland (luminal and stem/basal). Our preliminary results of this novel experiment suggest that at least in the context of Rb/p53 loss, the genetic lesion and not the cellular origin of transformation dictate tumor phenotype. Further experiments will allow us to determine whether one cell population is more susceptible to transformation and whether other types of mammary tumors are dependent upon the genetic lesion, cell of origin or a combination of both factors.

Finally, we have recently completed 2 high-throughput chemical screens on primary cell lines isolated from Rb/p53 deficient mammary tumors and are continuing to carry out genome-wide shRNA negative selection screens. The data generated from these screens will lead to the identification of novel therapeutic targets for the treatment of breast cancer.

REFERENCES

1. Jiang Z, Deng T, **Jones R**, Li H, Herschkowitz JI, Liu JC, Weigman VJ, Tsao MS, Lane TF, Perou CM, Zacksenhaus E. Rb deletion in mammary progenitors induces luminal-B or basal-like/EMT tumor subtypes depending on p53 status. *J Clin Invest* 2010 1;120(9):3296-3309.

2. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, Backlund MG, Yin Y, Khramtsov AI, Bastein R, Quackenbush J, Glazer RI, Brown PH, Green JE, Kopelovich L, Furth PA, Palazzo JP, Olopade OI, Bernard PS, Churchill GA, Van Dyke T, Perou CM. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol. 2007;8:R76.

3. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X and Perou CM. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010 12:R68.

4. Liu S and Wicha M. Targeting Breast Cancer Stem Cells. *J Clin Oncol* 2010 28(25): 4006-4012.

5. Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., and Eaves, C.J. *Nature* 2006. Purification and unique properties of mammary epithelial stem cells. 439:993-997.

6. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell Nature. 2006 5;439(7072):84-8.

7. Liu, J.C., Deng, T., Lehal, R.S., Kim, J., and Zacksenhaus, E.. Identification of tumorsphere- and tumor-initiating cells in HER2/Neu-induced mammary tumors. 2007 *Cancer Res.* **67**:8671-8681.

8. Cho, R.W., Wang, X., Diehn, M., Shedden, K., Chen, G.Y., Sherlock, G., Gurney, A., Lewicki, J., and Clarke, M.F. 2008. Isolation and molecular characterization of cancer stem cells in MMTV-Wnt-1 murine breast tumors. *Stem Cells* **26**:364-371.

9. Zhang M, Behbod F, Atkinson RL, Landis MD, Kittrell F, Edwards D, Medina D, Tsimelzon A, Hilsenbeck S, Green JE, Michalowska AM, Rosen JM. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. Cancer Res. 2008 Jun 15;68(12):4674-82.

10. Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, Knezevic J, Greene SB, Darr D, Troester MA, Hilsenbeck S, Medina D, Perou CM and Rosen JM. (2011) Comparative oncogenomics identifies breast tumors enriched in functional tumor initiating cells. Proc. Natl. Acad Sci, USA, June 1.

11. Guo W, Keckesova Z, Donaher JL, Shibue T, Tischler V, Reinhardt F, Itzkovitz S, Noske A, Zurrer-Hardi U, Bell G, Tam WL, Mani SA, van Oudenaarden A, Weinberg RA. Slug and Sox9 cooperatively determine the mammary stem cell state. Cell. 2012 Mar 2;148(5):1015-28

12. Alexandra Van Keymeulen, Ana Sofia Rocha, Marielle Ousset, Benjamin Beck, Gaëlle Bouvencourt, Jason Rock, Neha Sharma, Sophie Dekoninck, Cédric Blanpain. Distinct stem cells contribute to mammary gland development and maintenance**.** *Nature*, 2011 Nov (479) 189-193

Appendices-None

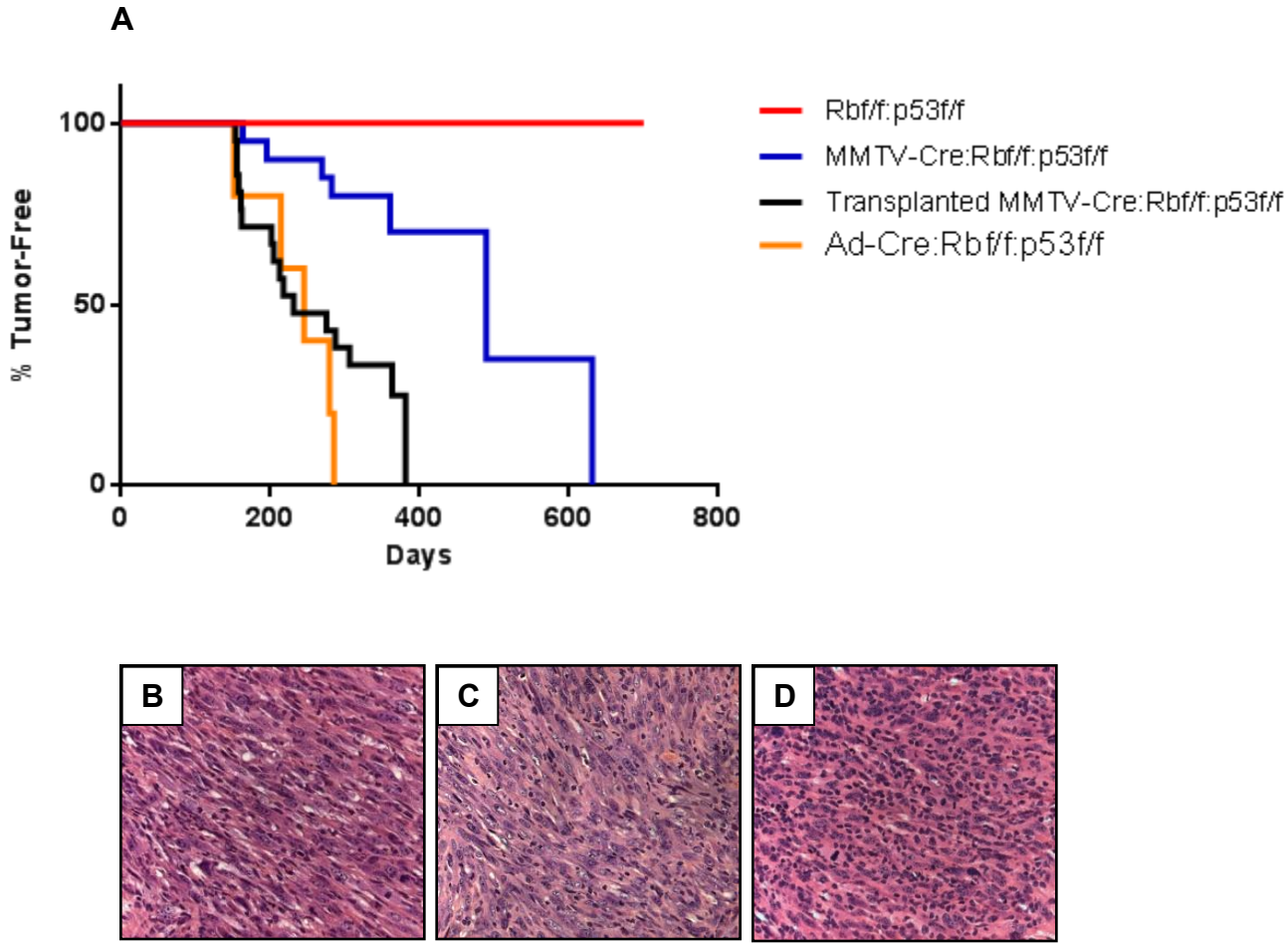
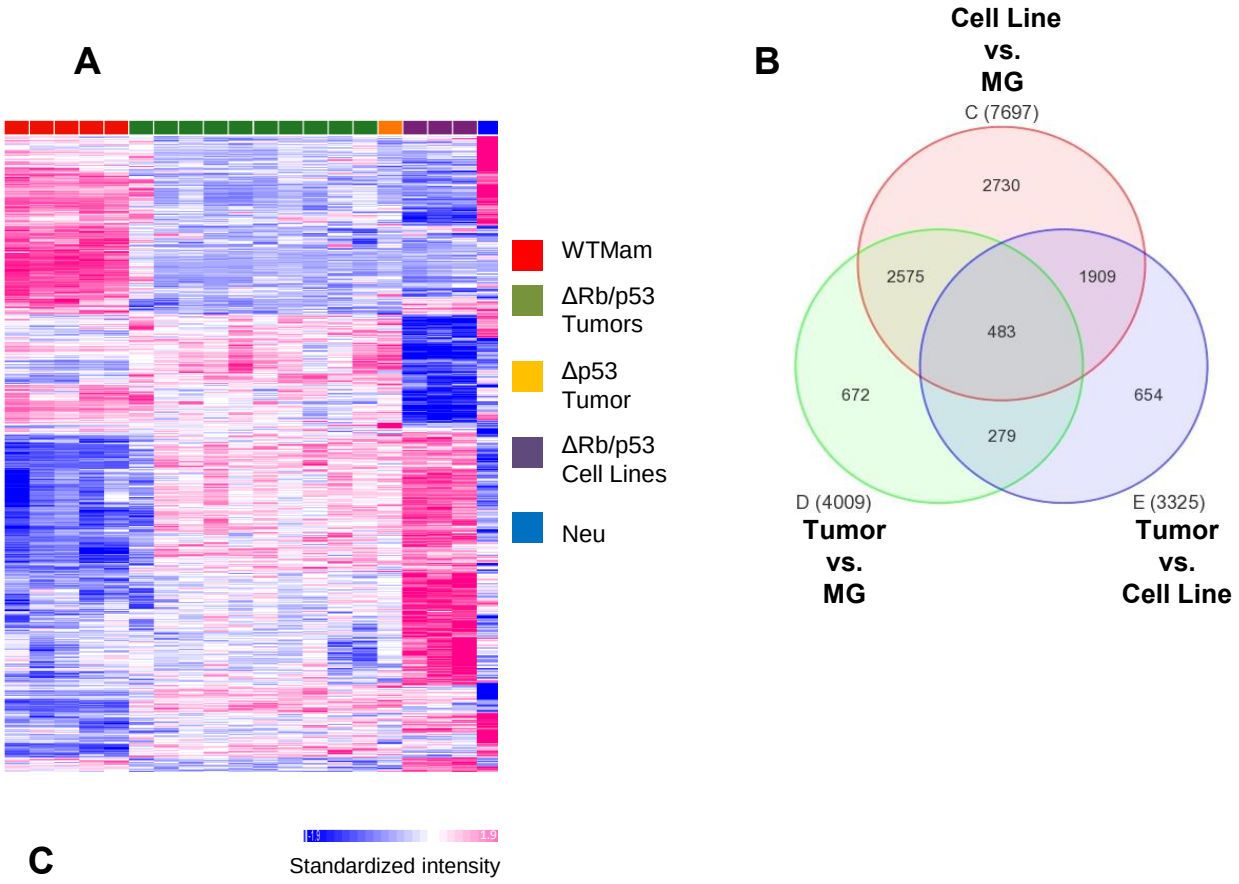


Figure 1. MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ double mutant mice develop spindle cell/EMT-type tumors similar to Rag1-/- mice transplanted with MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/f}$ cells . **(A)** Kaplan-Meier tumor-free survival curve of Rag1-/- animals transplanted with MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/f}$ cells (black), Rag1-/- mice transplanted with Ad-Cre infected $Rb^{fl/fl}$:$p53^{fl/f}$ cells (orange) and MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ (blue) and $Rb^{fl/fl}$:$p53^{fl/fl}$ (red) compound mutant mice. **(B-D)** Identical histology of mammary tumors that developed in **(B)** MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/fl}$ mice or following transplantation of **(C)** MMTV-Cre:$Rb^{fl/fl}$:$p53^{fl/f}$ cells or **(D)** Ad-Cre infected $Rb^{fl/fl}$:$p53^{fl/fl}$ cells into Rag1-/- recipients.



C

Gene Symbol	Gene Name	Fold-Change (Rb/p53 vs. MG)	Fold-Change (Rb/p53 CL vs. MG)
2310002L13Rik		19.0387	23.0301
Fam5c	family with sequence similarity 5, member C	17.637	10.5146
Mmp13	Matrix Metalloproteinase 13	13.9647	17.9858
Inhba	inhibin beta A	12.0388	9.21899
Ptgs2	Prostaglandin-endoperoxide synthase 2	11.7087	35.4274
Cyp2e1	Cytochrome P450 2E1	-47.6688	-103.757
Ear11	Eosinophil associated ribonuclease A	-50.5362	-32.2399
Adipoq	Adiponectin	-53.2015	-105.615
Tmem45b	Transmembrane 45B	-60.2809	-59.6805
Gys2	Glycogen synthase 2	-63.2357	-59.4552

Figure 2 . Microarray analysis of ΔRb/p53 tumors. **(A)** Unsupervised cluster analysis of normal mammary tissue (n=5), ΔRb/p53 tumors (n=10), Δp53 tumor (n=1), cell lines derived from ΔRb/p53 tumors (n=3) and an MMTV-Neu tumor (n=1). **(B)** Venn diagram illustrating genes that are shared between primary ΔRb/p53 tumors and ΔRb/p53 cell lines compared to WT mammary glands. **(C)** Table summarizing the top 5 upregulated and downregulated genes shared between ΔRb/p53 tumors and ΔRb/p53 tumor cell lines compared to WT mammary tissue.

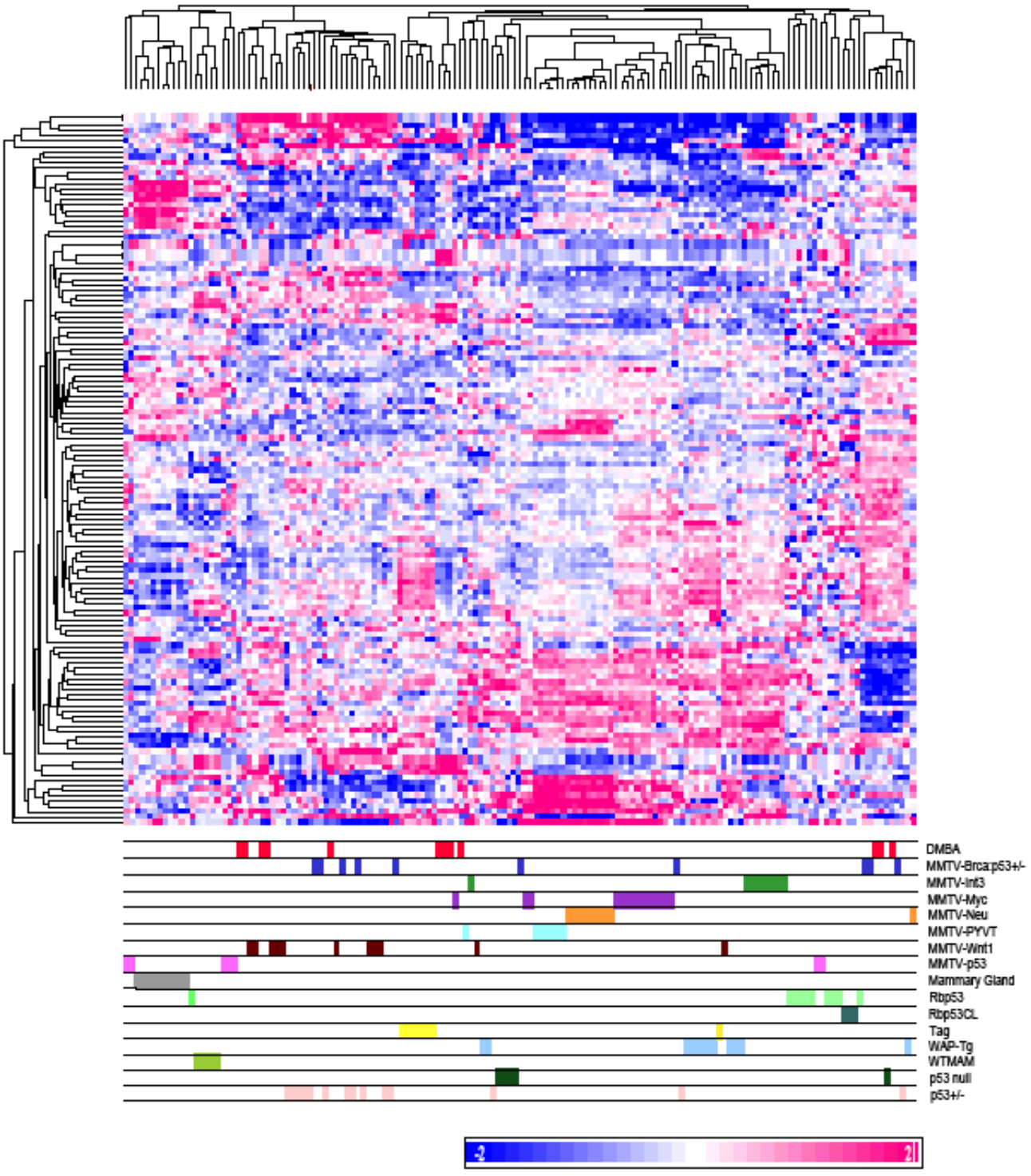


Figure 3. Comparison of ΔRb/p53 tumors with mouse models of breast cancer. Tumors induced following combined loss of Rb and p53 cluster with other mouse tumors that exhibit features of epithelial-mesenchymal transition including a subset of tumors from DMBA, MMTV-BRCA1;p53+/-, MMTV-Int3, p53+/-, p53 null and WAP-Tag mice. Expression levels are color coded from blue (low expression) to magenta (high expression) in relation to the row Z score across all tumors.

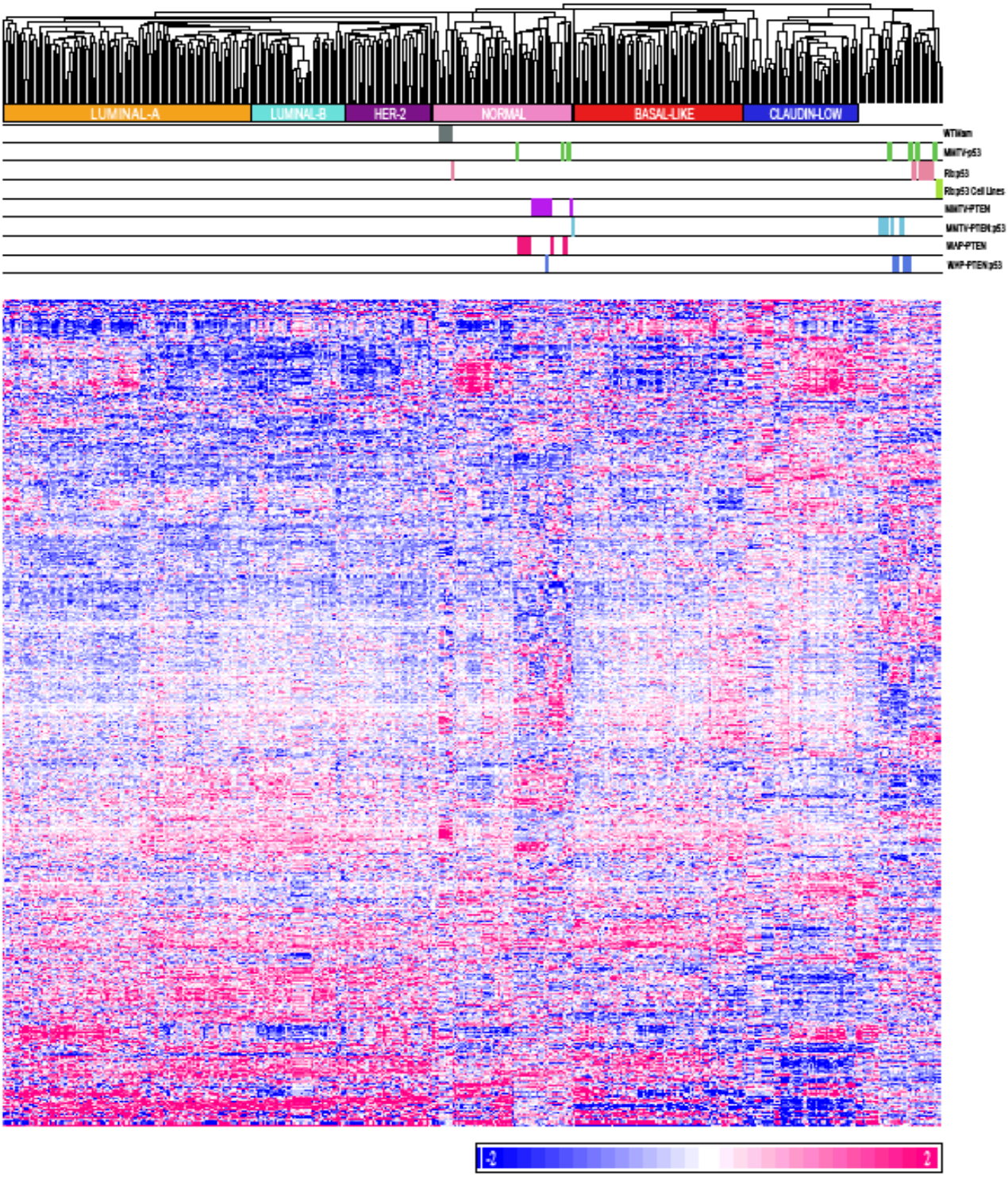
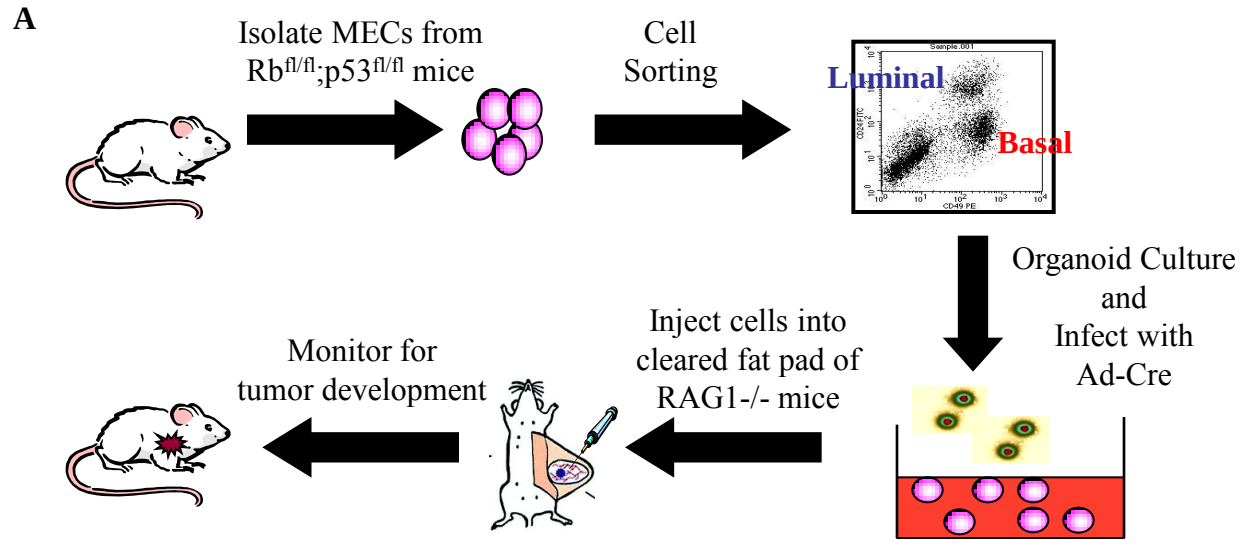
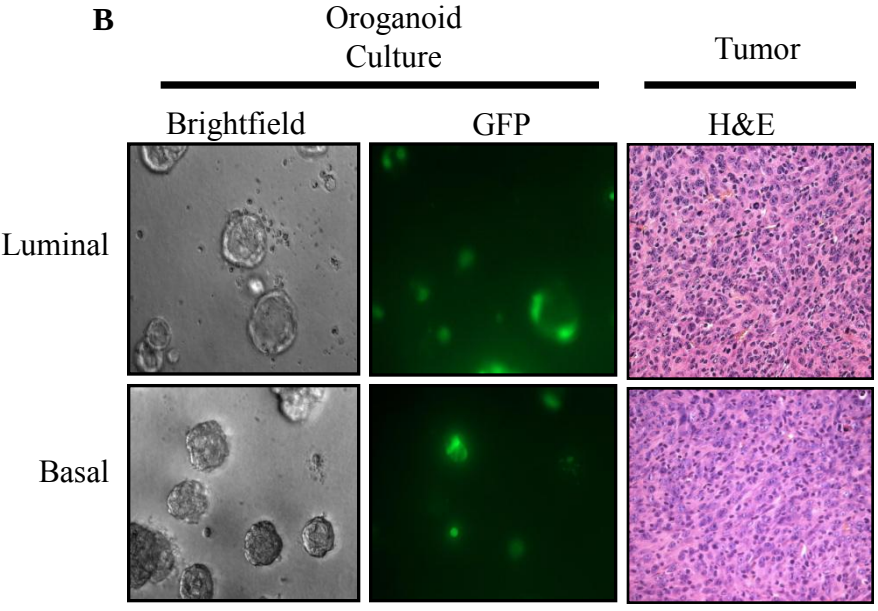


Figure 4. Comparison of ΔRb/p53 tumors with human breast cancer. ΔRb/p53 tumors cluster with human claudin-low tumors using the Claudin-Low predictor from Prat et al. (2010). Expression levels are color coded from blue (low expression) to magenta (high expression) in relation to the row Z score across all tumors.

# Cells	1000	250	100	50	10	TIC/Cells
Lin-CD24^{+}:CD49f^{+}	6/8	1/4	2/10	1/4	0/8	1/635 (1/443-883)
Lin-CD49f^{-}CD24^{+}	3/6	2/4	0/8	-	0/4	1/1174(1/740-1/1863)
Lin-CD49f^{+}: -CD24^{-}	7/8	2/4	6/10	4/4	4/8	1/217 (1/112-1/156)
Lin- CD49f^{-}:CD24^{-}	6/8	4/4	5/10	4/4	4/10	1/152 (1/192-1/268)
Lin-	2/2	2/2	2/2	-	-	-

Figure 5. Rb/p53 deficient tumors are enriched for tumor initiating cells. Limiting dilution transplantation analysis of Rb/p53 deficient tumors illustrating enrichment of tumor-initiating activity in Lin^{-}CD49^{-}:CD24^{-} and Lin-CD49f^{+}:CD24^{-} populations.





C

Experiment	Luminal (#mice/#tumors)	Basal
1	1/1	1/1
2	4/4	2/4
3*	0/3	1/3
4*	N/A	N/A

* experiment still in progress

Figure 6. (A) Schematic of experimental strategy used to investigate the cellular origin of Rb/p53 deficient tumors. Primary MECs were isolated from $Rb^{f/f};p53^{f/f}$ mice and separated into Luminal and Basal subpopulations by FACS using CD49f and CD24 cell surface markers. Cells were cultured in matrigel as organoids for 5-7 days, dissociated into single cells and then infected with Ad-Cre for 1hr. Approximately 10,000 cells from each subpopulation were then mixed with an equal number of Lin- wild type MECs (isolated from FvB or C57/Bl6 mice) and injected into the cleared fat pad of Rag-/- mice and monitored for tumor development. **(B)** Bright field and GFP expression of luminal and basal organoids grown from FACS sorted $Rb^{f/f};p53^{f/f}$ primary MECs and infected with Ad-Cre-GFP. Transplantation of Ad-Cre-GFP infected Luminal or Basal cells gave rise to tumors with an identical mesenchymal histology. **(C)** Table summarizing tumor incidence .

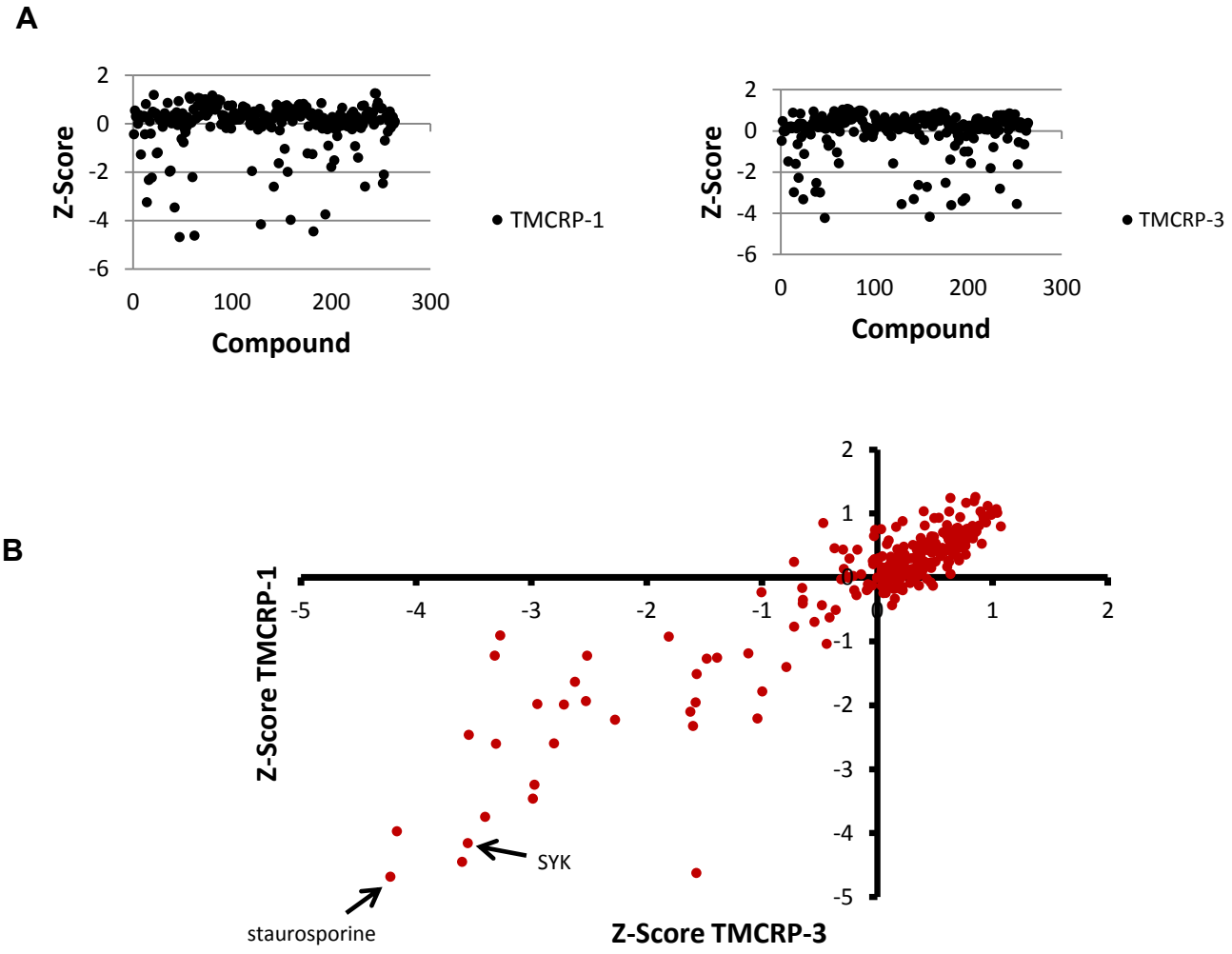


Figure 7. High-throughput small molecule kinase inhibitor screen on Rb/p53 deficinet mouse mammary tumor cells. (**A and B)** Example of scatter plots for 2 tumor cell lines screened with the library. **(C)** Scatter plot of the combined data from both cell lines to identify common 'hits'..

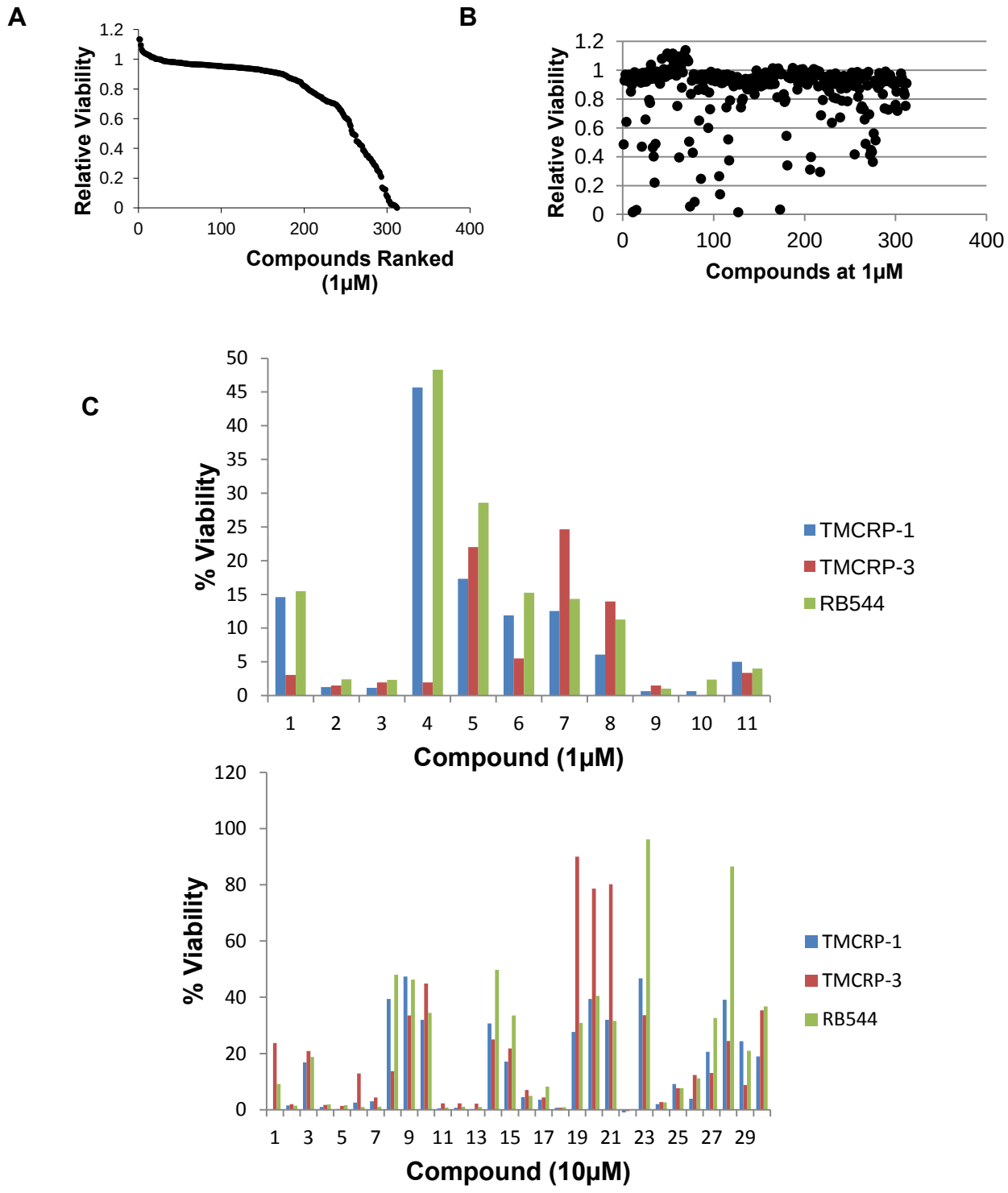


Figure. High-throughput FDA-approved pharmaceutical screen on Rb/p53 deficinet mouse mammary tumor cells . (**A)** Example of a rank-ordered plot and **(B)** scatter plot of the Sequoia library of 312 FDA-approved drugs showing their effects on the TMCRP-1 cell line. **(C)** Bar graphs illustrating compounds that reduced viability by at least 50% in at least 2 of 3 Rb/53 deficient mammary tumor cell lines treated with 1μM (Top) and 10μM (bottom) of the library.

ABSTRACT

The Second Internation Rb Meeting, Toronto, Canada

Role of the Rb and p53 Tumor Suppressor Pathways in Mammary Tumorigenesis

Robert A. Jones[1], Zhe Jiang[1], Tao Deng[1], Aaron D. Schimmer[1], Jason Moffat[2] and Eldad Zacksenhaus[1]

[1]University Health Network, [2]University of Toronto

The retinoblastoma (Rb) and p53 tumor suppressor pathways are frequently altered in human malignancies including breast cancer. We have recently reported that combined somatic loss of Rb and p53 in the mouse mammary epithelium led to the formation of tumors that often displayed features of an epithelial to mesenchymal transition (EMT) (Jiang et al., JCI 2010). We are now performing microarray and array-CGH analysis to correlate the molecular subtype and level of genomic instability with other mouse mammary tumor models as well as human breast cancers. In addition, using established cell surface markers of mouse mammary stem cells (CD49f and CD24) followed by limiting dilution transplantation analysis we have found that Rb/p53 deficient EMT-type tumours are enriched for tumor-initiating capacity. To investigate the cellular origin of Rb/p53 deficient tumors, the $Rb^{f/f}$ and $p53^{f/f}$ alleles will be deleted within the luminal, myoepithelial and stem/bipotent progenitor compartments of the mammary gland using a Cre-GFP expressing adenovirus and CD24-CD49f-based FACS analysis. The different cellular fractions will then be transplanted into the cleared mammary fat pad of recipient mice and tumors will be characterized at the histological, molecular and genetic level. Finally, we are using primary cell lines isolated from Rb/p53 deficient mammary tumors in conjunction with high-throughput chemical and genetic screens to identify new therapeutic targets. A primary screen of 260 kinase inhibitors and 312 FDA-approved off-patent drugs and a genome wide negative selection (‘drop-out’) screen is currently in progress. Ultimately, the results of this research will provide important insight into the biology of triple-negative breast cancer and will lead to the identification of novel therapeutic targets for the treatment of patients living with this disease.